Polymorphic Thiopurine Methyltransferase in Erythrocytes Is Indicative of Activity in Leukemic Blasts From Children With Acute Lymphoblastic Leukemia

By Howard L. McLeod, Mary V. Relling, Qing Liu, Ching-Hon Pui, and William E. Evans

The activity of thiopurine methyltransferase (TPMT) exhibits genetic polymorphism, with approximately 1 in 300 individuals inheriting TPMT deficiency as an autosomal recessive trait, and about 11% having intermediate activity (ie, heterozygotes). Patients with TPMT deficiency accumulate excessive concentrations of 6-thioguanine nucleotides (TGNs) and develop severe toxicity when treated with standard dosages of mercaptopurine. High TPMT activity has been associated with lower concentrations of TGNs, yielding a higher risk of treatment failure in children with acute lymphoblastic leukemia (ALL). As the biochemical basis of these pharmacodynamic relationships has not been fully elucidated, we investigated the variability and relationship of TPMT activity in erythrocytes and lymphoblasts from children with ALL. A 58-fold range of erythrocyte TPMT activity was found among 119 patients receiving ALL chemotherapy (0.6 to 34.9 U/mL packed erythrocytes), but relatively low intrapatient variability (coefficient of variation, 13.5%) was observed over 1 year. A 27-fold range in TPMT activity was observed in leukemic blasts obtained from 42 patients at initial diagnosis (3.3 to 88.9 U/1 × 10⁹ cells). TPMT activity in leukemic blasts at diagnosis was significantly correlated with TPMT in erythrocytes before therapy (rₓ = .75, P < .0001, N = 13). These data document extensive interpatient variability of TPMT activity in ALL blasts and establish its linkage to polymorphic TPMT activity in erythrocytes, providing a new mechanism by which erythrocytes serve as prognostic markers of mercaptopurine metabolism and TPMT activity in children with ALL.

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diagnosis, during continuation treatment, and at the cessation of therapy, as well as the extent of intrapatient variability in TPMT activity while chemotherapy is being administered.

MATERIALS AND METHODS

Patients and treatment. Erythrocyte TPMT activity was measured in patients with newly diagnosed acute lymphoblastic leukemia, before the initiation of chemotherapy, while remission chemotherapy was being given, and (or) after elective cessation of treatment after 2.5 years of chemotherapy. Leukemic blasts were isolated from bone marrow aspirates obtained at diagnosis, before initiation of chemotherapy. Blast cell immunophenotype, cytogenetics, and ploidy were determined as previously described.11 The correlation between erythrocyte and blast cell TPMT activity was assessed in all patients from whom both erythrocytes and leukemic blasts were available, and in the subset of children from whom both erythrocytes and leukemic blasts were obtained before start of ALL therapy. All patients were treated according to the same treatment protocol (TOTAL-XII), which included daily oral 6MP (75 mg/m²) and weekly intravenous or intramuscular methotrexate (40 mg/m²) for 2.5 years. During the first year of treatment, the 6MP and methotrexate therapy was interrupted every 6 weeks by pulse therapy with either high-dose methotrexate or teniposide plus cytarabine.12 The acute effect of teniposide plus cytarabine or high-dose methotrexate on TPMT activity was evaluated in 10 children by obtaining erythrocytes immediately before, at the end of infusion, and 24 hours after administration of these drugs. All samples were obtained at greater than 120 days after the most recent erythrocyte transfusion to avoid contamination of TPMT activity by donor erythrocytes. The study was approved by the Institutional Review Board for Clinical Trials at St. Jude Children's Research Hospital (Memphis, TN), and informed consent was obtained from the patients or their guardians.

Sample collection and preparation. Samples for erythrocyte TPMT were obtained during clinic visits, while blood was being drawn as part of standard patient management. Venous blood (5 mL) was collected into tubes containing sodium heparin. Samples were stored at 4°C until processed. All samples were processed within 24 hours after they were obtained. Erythrocytes were washed in triplicate with 0.9% NaCl and lysed with cold water. Erythrocyte membranes were then separated by centrifugation for 10 minutes at 12,000g. The lysates were stored at −80°C until analyzed.

Bone marrow samples were obtained at diagnosis, before initiation of chemotherapy. Mononuclear cells were separated by centrifugation on a Ficoll-Hypaque density gradient, erythrocytes were lysed in water, and blasts were washed in triplicate with 0.9% NaCl. ALL blasts (1 × 10⁶) were centrifuged at 7,000g for 10 minutes at 4°C. The pellet was resuspended in 1 mL 5 mmol/L Tris-HCl buffer, pH 7.8 (containing 7 mg/mL bovine serum albumin). Cells were homogenized for 30 seconds and centrifuged at 100,000g for 1 hour at 4°C. The supernatant was stored at −80°C until analyzed.

TPMT assay. The erythrocyte lysates were analyzed for TPMT activity by the nonchelated radiochemical assay of Szumianski et al.1 This modification allows more rapid sample analysis but yields activity values 15.6% higher than the original chelated assay.13 Erythrocyte TPMT values from previously published studies using the chelated assay must, therefore, be adjusted 15.6% for comparison to enzyme activity in this study. The assay is based on the conversion of 6MP to radioactively labeled MeMP with S-[methyl-14C]-adenosyl-L-methionine as the methyl donor. One unit of enzyme activity represents the formation of 1 nmol of MeMP per hour of incubation. TPMT activity was normalized per milliliter of packed erythrocytes (pRBC). In our laboratory, the coefficient of variation (CV) for the erythrocyte TPMT assay was 4.6% for multiple assays of the same sample (n = 20; mean activity, 12 U/mL pRBC).

Bone marrow blast cells were analyzed for TPMT activity by a modification of a previously described radiochemical assay.1 Blast lysate (100 μL) was incubated with 7.5 mmol/L 6MP, 15 mmol/L glutathione, 50 μmol/L allopurinol, 1 mol/L potassium phosphate pH 7.5, and 23 μmol/L (24 μCi/μmol) S-[methyl-14C]-adenosyl-L-methionine for 1 hour (concentrations are those in final volume of 160 μL). Radioactively labeled MeMP was extracted with 20% isooamyl alcohol in toluene. Results were normalized to 1 × 10⁶ blasts, based on the number of cells extracted for the 100 μL of lysate. The use of reduced glutathione as the sulfhydryl donor gave 24% greater lymphocyte TPMT activity than dithiothreitol. In our laboratory, the CV for lymphoblast TPMT assay was 8% for multiple assays of the same lymphocyte sample (n = 10; mean activity, 56 U/1 × 10⁶ cells).

Chemicals and reagents. S-[methyl-14C]-adenosyl-L-methionine (specific activity, 58 μCi/mmol) was purchased from New England Nuclear Research Products (Boston, MA). S-adenosyl-L-methionine (chloride salt), DL-dithiothreitol, glutathione, allopurinol, dimethyl sulfoxide, tris(hydroxymethyl)aminomethane, bovine serum albumin, and 6MP were obtained from Sigma Chemical Co (St Louis, MO). Bio-Safe II scintillation cocktail was obtained from Research Products International Corp (Mount Prospect, IL). Lymphoprep Ficoll-Hypaque solution was obtained from Nycomed (Oslo, Norway).

Data analysis. Multiple regression was used to investigate the relationship between erythrocyte TPMT activity and sex, race, and age at diagnosis. Intraindividual changes in erythrocyte TPMT activity were addressed by an exact Wilcoxon signed rank test. The Spearman correlation coefficient was also used to assess the relation of pretreatment erythrocyte TPMT and the percent increase once therapy was started, and the relation between on-therapy erythrocyte TPMT and the percent decrease after cessation of therapy. The influence of blast cell cytogenetics and leukemic phenotype on blast TPMT activity was examined by the Kruskal-Wallis test. Association between erythrocyte and blast TPMT activity was measured by the Spearman’s correlation coefficient (r_s). Wilcoxon rank sum test was used to compare the blast TPMT activity between patients with and without erythrocyte TPMT deficiency. Linear regression was also used to assess the relation between erythrocyte and blast TPMT activity in two subsets of the study population, those with pretreatment erythrocyte TPMT (n = 13) or those with on-therapy erythrocyte TPMT activity (n = 27), versus TPMT activity in ALL blasts at diagnosis. For patients with multiple measurements of TPMT activity in erythrocytes while chemotherapy was being given, the median of all values was used in the analysis.

RESULTS

Leukemic blasts were isolated from bone marrow samples at diagnosis from 42 children with ALL, aged 1.5 to 16.6 years (median, 4.9 years). In the children from whom ALL blasts were obtained at diagnosis, 13 also had erythrocytes obtained before therapy and before any erythrocyte transfusions. Erythrocytes were obtained from an additional 139 children with ALL (70 girls, 69 boys) aged 1 to 21.6 years (median, 6 years); 119 were studied on-therapy and 20 only after chemotherapy had been completed. Of the 119 studied on-therapy, 10 were also studied before therapy, and 30 were also studied 2 to 18 months after completion of chemotherapy. A single erythrocyte sample was obtained from 53 patients on-therapy (44.5%); while two, three, or four erythrocyte samples were obtained on-therapy in 39 (32.8%), 25 (21%), and two (17%) children, respectively.

Patient characteristics and erythrocyte TPMT activities are
summarized in Table 1. Age, sex, or race was not significantly related to erythrocyte TPMT activity in this study. There was a 58-fold range in erythrocyte TPMT activity among 119 children studied on-therapy, with two children having activity consistent with TPMT deficiency (less than 1 U/mL pRBC), as previously described in detail. As shown in Fig 1, erythrocyte TPMT activity in patients receiving continuation chemotherapy was greater than activity measured after cessation of therapy (median, 21.2 ± 15.1 U/mL pRBC). Erythrocyte TPMT activity increased after initiation of chemotherapy in 9 of 10 patients studied, increasing a median of 33.6% (range, 4.3% to 47.2% change; Wilcoxon rank-sum test, P = .002; Fig 2). There was a significant correlation between the pretreatment TPMT activity and the percent increase (r = −.927; P = .0001), with the greatest increase in activity occurring in patients with the lowest pretreatment values. Erythrocyte TPMT activity decreased after the cessation of 6MP therapy in 28 of 30 children, declining a median of 32.9% (range, 5.1% increase to 55.7% decrease; Wilcoxon rank-sum test, P < .0001; Fig 2). Again, there was a significant correlation between the TPMT activity on-therapy and the percent decrease (r = .64; P = .0001), with the greatest decrease in patients with higher erythrocyte TPMT activity during therapy. In these 30 patients, hemoglobin concentrations increased a median of 13% (range, 1.5% to 33.3%) from the on-therapy to the off-therapy study points, but the percentage change in TPMT activity was not correlated with the percentage change in hemoglobin concentration (r = .24).

Intraventricular variability of erythrocyte TPMT activity was evaluated in the 27 children from whom three or four samples were obtained over a 1-year period of therapy. While patients were receiving remission chemotherapy, erythrocyte TPMT activity demonstrated relatively low intrapatient variability over 1 year, with a median CV of 13.5% (range, 4.0% to 24.4%). The length of time patients had been receiving 6MP treatment was not correlated with TPMT activity.

The acute effects of teniposide plus cytarabine or high-dose methotrexate chemotherapy on erythrocyte TPMT activity were evaluated in two groups of 10 children receiving either teniposide plus cytarabine intravenously over 4 hours or high-dose methotrexate intravenously over 24 hours. Erythrocyte TPMT activity ranged from 5.1 to 24.5 and 10.2 to 19.8 U/mL pRBC before teniposide plus cytarabine or high-dose methotrexate, respectively. Erythrocyte TPMT was not significantly different from pretreatment values either at the end of infusion or 24 hours after administration of either teniposide plus cytarabine (median change at 24 hours post-therapy, 5.0%) or high-dose methotrexate (median change at 24 hours post-therapy, 6.5%).

Table 2 is a summary of the demographic and clinical characteristics of patients from whom bone marrow ALL blast cells were obtained at diagnosis. Blast TPMT activity varied by 27-fold (range, 3.3 to 88.9 U/1 × 10^6 cells; Fig 3). Patient age, sex, race, white blood cell count at diagnosis,
percent cells in S phase, or cytogenetics of leukemic blasts was not related to blast TPMT activity. Blast TPMT activity was significantly lower in the two patients with inherited erythrocyte TPMT deficiency (P < .01, one-sided Wilcoxon rank sum test). When the two patients with congenital TPMT deficiency were excluded, there was a sevenfold range of ALL blast TPMT activity (12.5 to 88.9 U/1 × 10⁹ cells). In these 40 patients, TPMT activity tended to be higher in pre-B-cell and T-lineage blasts compared with blasts from patients with early pre-B-cell ALL; however, the difference did not reach the conventional level of statistical significance (median: 32.6, 27.8, and 20.9 U/1 × 10⁹ cells, respectively; P = .09, Kruskal-Wallis). There was not a significant correlation between TPMT activity measured in leukemic blasts before therapy (ie, at diagnosis) and TPMT activity measured in erythrocytes while chemotherapy was being given (r² = .01; P > .8). However, as shown in Fig 4, there was a significant association between TPMT activity in ALL blasts and erythrocytes when both were measured at diagnosis, before the initiation of chemotherapy (r = .75; P < .0001; N = 13). There was a significant linear correlation between ALL blast and erythrocyte TPMT activity at diagnosis when only the 13 patients without erythrocyte TPMT deficiency were assessed (r² = .48; P < .009; N = 13) or when the two patients with erythrocyte TPMT deficiency were also included (r² = .51; P < .005; N = 15).

**DISCUSSION**

Variability in the pharmacokinetics of many anticancer drugs has been identified as a potentially important factor influencing their toxic effects and therapeutic efficacy.³⁴ For 6MP, high erythrocyte concentrations of TGNs have been associated with greater hematopoietic toxicity,⁹,¹⁰ while low erythrocyte TGN concentrations have been associated with a greater risk of relapse in children with ALL.⁸,¹⁵,¹⁶ As 6MP constitutes a substantial component of ALL treatment, it is important to identify patient and treatment variables that influence the disposition and effects of 6MP. Previous studies have demonstrated that erythrocyte TPMT activity is a significant determinant of TGN concentrations in erythrocytes, with higher TPMT activity associated with lower erythrocyte TGN concentrations, presumably because less parent drug is available for activation to TGN. Conversely, patients with inherited TPMT deficiency accumulate excessive TGN concentrations, necessitating a substantial (eg, 8- to 15-fold) dosage reduction to avoid severe toxicity.⁹,¹⁰,¹⁷ The biochemical basis for erythrocyte TPMT activity and TGN concentrations being of prognostic importance for toxicity and efficacy has not been established. Previous studies have shown that erythrocyte and hepatic TPMT activity are significantly correlated; thus, erythrocyte TPMT may be indicative of the systemic metabolism of 6MP. Increased hepatic methylation of 6MP could result in less 6MP for uptake by leukemic blasts and subsequent intracellular activation to TGN. Thus, high hepatic TPMT activity could explain the greater risk of ALL treatment failure in children with high erythrocyte TPMT. However, the present study indicates that

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**Table 2. Patient Characteristics and Leukemic Cell TPMT Activity in Patients From Whom ALL Blasts Were Obtained at Diagnosis**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>42</td>
</tr>
<tr>
<td>Median age, yrs (range)</td>
<td>5.3 (1.5-16.6)</td>
</tr>
<tr>
<td>Sex (F:M)</td>
<td>18:24</td>
</tr>
<tr>
<td>Race (W:B:H)</td>
<td>38:3:1</td>
</tr>
<tr>
<td>Median WBC count at diagnosis, x1,000 cells/μL (range)</td>
<td>22.6 (2-581)</td>
</tr>
<tr>
<td>Median % cells in S phase (range)</td>
<td>6.2 (0.6-18.3)</td>
</tr>
<tr>
<td>ALL ploidy</td>
<td></td>
</tr>
<tr>
<td>Pseudodiploid</td>
<td>20</td>
</tr>
<tr>
<td>Hyperdiploid</td>
<td>17</td>
</tr>
<tr>
<td>Diploid</td>
<td>5</td>
</tr>
<tr>
<td>ALL immunophenotype</td>
<td></td>
</tr>
<tr>
<td>Early pre-B-cell</td>
<td>23</td>
</tr>
<tr>
<td>Pre-B-cell</td>
<td>9</td>
</tr>
<tr>
<td>T-lineage</td>
<td>10</td>
</tr>
<tr>
<td>Median TPMT activity, U/1 × 10⁹ cells (range)</td>
<td>17.6 (3.3-88.9)</td>
</tr>
</tbody>
</table>

Abbreviations: H, Hispanic; WBC, white blood cell.
erythrocyte TPMT may also be of prognostic importance in that it is indicative of TPMT activity within the pharmacologic target itself: leukemic lymphoblasts. Thus, high lymphoblast TPMT activity would further decrease the amount of 6MP available for anabolism to active TGNs within leukemic cells.

This study represents the first report of TPMT activity in leukemic lymphoblasts obtained by bone marrow aspirates from patients with ALL. A 27-fold range in blast TPMT activity was observed among 42 children whose leukemic blasts were studied at initial diagnosis, before therapy. The substantial variability in blast TPMT activity indicates that this could contribute to differences in ALL blast sensitivity to 6MP. Previous studies of 6MP resistance have focused on HPRT, the initial enzyme for 6MP metabolism to TGN. Pieters et al. described a 10-fold range in HPRT activity in blast cells from 83 children with ALL, with lower activity in T-lineage ALL compared with B-lineage blasts and a poorer prognosis for patients with HPRT activity below the median value. By contrast, no clear association between blast TPMT activity and ALL immunophenotype, cytogenetics, or ploidy was observed in the present study, although there was a trend toward higher activity in T-lineage blasts. However, TPMT activity in ALL blasts at diagnosis was significantly associated with activity in erythrocytes obtained before therapy, providing a feasible method of estimating blast TPMT activity and an additional mechanism for the previously reported prognostic importance of erythrocyte TGN concentration in children with ALL.

Previous studies have established a correlation between erythrocyte TPMT activity and that measured in normal lymphocytes, platelets, kidney, and liver tissue; the present study extends this linkage to leukemic blasts. However, it should be noted that TPMT activity in ALL blasts obtained before the start of chemotherapy did not correlate with TPMT in erythrocytes obtained after chemotherapy had been initiated ($r^2 = .01; P > .8$). This is not surprising, as erythrocyte TPMT activity consistently increases after chemotherapy is initiated and, thus, may not reflect constitutive TPMT activity in ALL blasts before chemotherapy. It is plausible that the increase in erythrocyte TPMT activity that occurs after the initiation of chemotherapy also occurs in leukemic blast cells; however, this cannot be readily addressed in vivo, as no ALL blasts are available for study once patients are in complete remission. (Yet it is estimated that greater than $10^9$ leukemia cells remain in patients who achieve a complete remission, necessitating 2.5 years of additional chemotherapy.)

In the present study, erythrocyte TPMT activity in 119 children evaluated on-therapy had a median value similar to previous reports in children with ALL on-therapy (21.2 v 19.0 U/mL pRBC), exhibited large interpatient differences (ie, 58-fold range), and had a distribution of activity consistent with the genetic polymorphism previously reported. An increase in erythrocyte TPMT activity after initiation of ALL chemotherapy was consistently observed within individual patients in this study. Moreover, we demonstrated that pretreatment erythrocyte TPMT activity was similar to erythrocyte TPMT activity after the cessation of therapy. Throughout chemotherapy, erythrocyte TPMT activity was relatively stable, with a median CV of 13.5% over 1 year, indicating that multiple measurements of TPMT activity should not be required to adjust 6MP therapy in individual patients. However, the present study indicates that a single measurement of erythrocyte TPMT activity before starting chemotherapy will not necessarily be representative of erythrocyte TPMT activity once treatment has been initiated, as there was considerable heterogeneity in the percent increase in erythrocyte TPMT after treatment was initiated (−4% to +47% change; median, 33.6% increase).

TPMT activity consistently decreased after cessation of therapy, duplicating the 32% decrease observed in a previous study and comparable to the 33.6% increase observed with the initiation of therapy in the present study. The decrease in activity was observed as early as 2 months postchemotherapy, although the precise relationship between time postchemotherapy and decrease in TPMT activity was not assessed in this study. Erythrocyte TPMT activity in the 50 children who were studied 2 to 18 months after cessation of chemotherapy was similar to previous reports in children with ALL after chemotherapy had been completed (median, 15.1 v 14.6 U/mL pRBC), and also similar to previous findings from our laboratory in 209 healthy, white adult blood donors (median, 15.1 v 16.8 U/mL pRBC). Collectively, these data indicate that an increase in erythrocyte TPMT activity occurs after initiation of ALL remission therapy with 6MP and methotrexate and returns to pretreatment levels after cessation of chemotherapy. There was not a significant relationship between the percentage change in hemoglobin and the change in TPMT activity, indirect evidence that TPMT activity was not a function of red cell age. Additional studies will be required to define the mechanism(s) underlying higher erythrocyte TPMT activity during chemotherapy. Recent isolation of a cDNA encoding human TPMT and identification of an inactivating point mutation at the human TPMT locus, provide insights to the molecular basis for this genetic polymorphism and regulation of TPMT activity.

Lennard et al. have demonstrated that high TPMT activity is associated with low erythrocyte TGN concentrations, and that low TGN is associated with greater risk of treatment failure (ie, relapse) in children with ALL. However, Bostrom and Erdmann recently reported that TPMT activity was lower in ALL patients who relapsed. It is unclear whether these apparently disparate results are caused by lower TPMT in relapsing patients secondary to noncompliance with chemotherapy, as apparently lower TPMT activity in children with ALL, and that pretreatment erythrocyte TPMT activity may
be a useful surrogate for pretreatment blast TPMT activity. While 6MP metabolism by TPMT decreases the formation of active TGNs by a competing pathway, it increases the formation of MeMPNs, metabolites that are cytotoxic by an alternative mechanism (ie, inhibition of de novo purine synthesis). As the relative importance of these two metabolites is not known, additional studies are needed to determine the extent to which ALL blast TPMT activity influences the sensitivity or resistance of leukemia cells to 6MP therapy in vivo. Such data may make it possible to more precisely determine the optimal intracellular concentrations of TGN and MeMPN, thereby providing improved guidelines for determining the optimal dosage and schedule of 6MP for individual patients with ALL.

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